A METHOD FOR SNP (single nucleotide polymorphism) TYPING

FIELD OF THE INVENTION

The present invention relates to a method for single nucleotide polymorphism (so-called "SNP") typing which is used for identifying polymorphism of a site of SNP in genomic DNA.

BACKGROUND OF THE INVENTION

Just as human appearances vary widely, their 3 billion genetic codes differ at numerous sites when compared between individuals. Such differences in genetic codes are called polymorphisms. A single nucleotide polymorphism (hereinafter referred to as "SNP") is known as a typical polymorphism.

SNP, single nucleotide polymorphism, means a single base difference among a plurality of individuals. SNPs are classified into cSNP (coding SNP) and gSNP (genome SNP) according to their location. cSNP further includes sSNP (silent SNP), rSNP (regulatory SNP) and iSNP (intron SNP). In this specification, a site at which SNP occurs in genomic DNA, that is, a certain nucleotide at which SNP occurs is referred to as "single nucleotide polymorphism site" or "SNP site."

Three to ten million SNP sites are thought to exist in the human genome. It is thought that some of these SNPs affect the control of expression or functions of proteins, and some involve individual differences in body compositions and susceptibility to a disease. That is, made to order medical care can be given according to an individual's body composition by obtaining information on SNPs. Accordingly, SNPs are increasingly discovered and identified, and many SNPs have already been reported.

The next task is to analyze information about how each SNP affects responsiveness to drugs and diseases, body composition or the like. To accomplish the task, SNP typing (a process to discriminate nucleotides of SNP sites) must be done to know how each individual's SNPs are. Such SNP typing would be a large-scale analytical process, such that SNP typing is performed for several hundreds of thousands of SNP sites per individual.

Examples of SNP typing are those using genomic DNA, including a TaqMan PCR method, an invader assay, a SniPer method, a MALDI-TOF-MASS method, a DNA chip method and the like. All of these methods require at least several tens of nanograms of genomic DNA to genotype one SNP site. When several hundreds of thousands of SNP sites are genotyped, several mg of genomic DNA is required per individual.

Nearly 1l of blood must be collected from an individual to obtain several mg of genomic DNA, however, it is actually impossible to obtain genomic DNA in such a volume from an individual. That is, typing of hundreds of thousands of SNP sites per individual cannot be performed at one time.

SUMMARY OF THE INVENTION

We have completed the present invention under these circumstances. The purpose of the present invention is to provide a method for SNP typing which can genotype hundreds of thousands of SNP sites using a remarkably small amount of genomic DNA.

A method for SNP typing according to the present invention, which has achieved the above purposes, comprises the steps of simultaneously amplifying a plurality of nucleotide sequences comprising at least one or more SNP sites using genomic DNA

and a plurality of primer pairs; and typing to discriminate nucleotides of SNP sites contained in the plurality of the nucleotide sequences amplified by the above amplification step.

In the method for SNP typing according to the present invention, a polymerase chain reaction using a hot start method is preferably used in the above amplification step.

Further in the method for SNP typing according to the present invention, 50 or more primer pairs are preferably used in the above amplification step.

Furthermore in the method for SNP typing according to the present invention, an Invader assay or a TaqMan PCR method is preferably used in the above typing step.

Furthermore in the method for SNP typing according to the present invention, an amplification is carried out from 10 ng to 40 ng of genomic DNA.

DETAILED DESCRIPTION OF THE INVENTION

Now the method for SNP typing according to the present invention will be further described in detail.

In the method for SNP typing according to the present invention, first, an amplification step is performed to simultaneously amplify a plurality of nucleotide sequences comprising at least one or more SNP sites using genomic DNA to be analyzed and a plurality of primer pairs. Then a typing step is performed for typing using the nucleotide sequences amplified in the amplification step.

1. Amplification step:

In the method of the present invention, genomic DNA to be analyzed can be extracted by using standard, known techniques. Genomic DNA to be analyzed can also be extracted by isolating leucocytes from peripheral blood collected from a human, and extracting according to standard techniques from the isolated leucocytes. Particularly when hundreds of thousands of SNP sites are genotyped, approximately several tens of micrograms of genomic DNA is prepared in the method of the invention. In other word, the method of the invention can perform typing of hundreds of thousands of SNPs from several ml of peripheral blood.

In the amplification step, a so-called multiplex polymerase chain reaction (multiplex PCR) is performed using genomic DNA prepared as a template DNA and a plurality of primer pairs for amplifying nucleotide sequences containing SNP sites to be genotyped. A preferred plurality of primer pairs are designed so as to be able to amplify 100 to 1500 bp DNA fragments flanking an SNP site. Each of these primers preferably comprises 17 to 25 nucleotides, more preferably, 18 to 22 nucleotides, respectively. These primers are designed so as to flank an SNP site to be genotyped, based on, for example, nucleotide sequence information accumulated in a database such as the GenBank.

The amplification step is performed by thermally denaturing template DNA (genomic DNA), followed by repetition of a cycle consisting of a thermal denaturation process to denature the template DNA, an annealing process to precisely anneal a plurality of primers, and an extension process to synthesize a DNA strand from the annealed primers. Finally, another extension step is performed to further extend the DNA strand. In addition, an appropriate temperature and time are preferably set separately for each process.

In such an amplification step, a preferred amount of a template DNA is 10 to 40 ng when, for example, nucleotide sequences of 100 regions are amplified using 100

pairs of primers. When the amount of a template DNA is less than 10 ng, amplifying all 100 regions would be difficult. In other words, with 10 ng or more of a template DNA, DNA fragments can be amplified in an amount sufficient to perform the typing step described later. When the amount of template DNA exceeds 40 ng, it becomes impractical to genotype hundreds of thousands of SNP sites because a large amount of genomic DNA is required.

In such an amplification step, a so-called hot start method is preferably applied. The hot start method is a technique in which the extension reaction with DNA polymerase is started when a reaction solution reaches a temperature high enough to prevent an annealing error and dimerization of primers. Examples of the hot start method include a method in which at least one kind of composition essential for PCR is added only when a reaction solution reaches a high temperature, a method which uses a wax barrier, and a method which uses a monoclonal antibody for DNA polymerase.

In the method using a wax barrier, first in a reaction container, solid wax divides an upper layer solution containing DNA polymerase and template DNA from a lower layer solution containing a primer and dNTP. Subsequently, PCR is allowed to proceed only after wax is melted by heating to a certain temperature so as to mix the upper and lower layer solutions.

In the method using a monoclonal antibody for DNA polymerase, DNA polymerase and a monoclonal antibody are bound to each other, and thus DNA polymerase is inactive until a reaction solution reaches a certain temperature. When the reaction solution is heated to a certain temperature (approximately 70℃ or more), the monoclonal antibody is irreversibly, thermally denatured and released from DNA polymerase. Thus, DNA polymerase is activated and PCR proceeds.

In any of these methods, the hot start method can prevent extension reaction from

proceeding where annealing errors occur for each primer, or dimerization of primers to each other. Therefore, amplification of undesired DNA fragments can be prevented.

When the above-mentioned hot start method is applied in the amplification step, nucleotide sequences of 300 or more regions can be amplified simultaneously using 300 or more primer pairs. Hence, the method of the present invention enables typing of a greater number of SNPs, because application of the above hot start method allows amplification of a greater number of nucleotide sequences at one time.

2. Typing Step:

The typing step is a process to genotype a plurality of SNPs using DNA fragments amplified in the above-mentioned amplification step. In the method of the present invention, typing can be performed, for example, by applying a TaqMan PCR method or an Invader assay using the DNA fragments obtained in the amplification step.

The TaqMan PCR method utilizes PCR using a fluorescence-labeled allele-specific oligonucleotide (hereinafter, referred to as TaqMan probe), a template DNA containing an SNP to be genotyped, and Taq DNA polymerase (Livak, K. J. Genet. Anal. 14, 143 (1999); Morris T. et al., J. Clin. Microbiol. 34, 2933 (1996)). A TaqMan probe is designed based on SNP information, and which has a 5' end labeled with fluorescent reporter dye R, such as FAM or VIC, and a 3' end labeled with quencher Q (Fig. 1) at the same time. Since, in this condition, the quencher absorbs fluorescence energy, fluorescence from the TaqMan probe cannot be detected. Further, since the 3' end of the TaqMan probe is phosphorylated, no extension reaction occurs from the TaqMan probe during PCR reaction (Fig. 1).

The following reactions occur when PCR is performed for a template DNA using the above described TaqMan probe, primers designed to amplify a region containing

SNP site, and Taq DNA polymerase. First, the TaqMan probe hybridizes to a sequence specific to the template DNA (Fig. 2a), while extension reaction occurs from the PCR primer hybridizing to the template DNA (Fig. 2b). At this time, Taq DNA polymerase having 5' nuclease activity cleaves nucleotides containing fluorescent reporter dye R of the TaqMan probe when extension reaction from PCR primers reaches the 5' end of the TaqMan probe (Fig. 2c). When a nucleotide containing fluorescent reporter dye R is cleaved as described above, the fluorescent reporter dye R becomes unaffected by quencher Q, and emits a measurable fluorescence signal. Hence, detection of fluorescence of the fluorescent reporter dye R by a fluorescent detector enables confirmation of hybridization of the TaqMan probe and the template DNA.

For example, as shown in Fig. 3, an SNP site is supposed to be present at A in allele 1(supposed to be allele 1) and that at G in allele 2 (supposed to be allele 2). While a TaqMan probe specific to allele 1 is labeled with FAM, a TaqMan probe specific to allele 2 is labeled with VIC (Fig. 3). The two types of TaqMan probe are added to PCR reagent, and then TaqMan PCR is performed for a template DNA containing SNP to be genotyped. PCR causes a nucleotide containing fluorescent reporter dye R to be released from a TaqMan probe having a nucleotide sequence complementary to SNP site to be genotyped, so that fluorescence is emitted. Then, fluorescent intensity of FAM and that of VIC are measured using a fluorescent detector.

As a result, SNP in the template DNA can be genotyped as a homozygote of allele 1 when strong fluorescence of FAM and almost no fluorescence of VIC are detected with a fluorescent detector. SNP in a template DNA can also be genotyped as a heterozygote of allele 1 and allele 2 when fluorescence of both FAM and VIC is detected with a fluorescent detector. Further, SNP in the template DNA can be genotyped as a homozygote of allele 2 when strong fluorescence of VIC and almost no fluorescence of FAM are detected with a fluorescent detector.

On the other hand, the Invader assay uses DNA comprising the SNP to be genotyped, two types of reporter probes specific to each allele of SNP to be genotyped, one type of Invader probe, and enzyme having special endonuclease activity to cleave by recognizing DNA structure (Livak, K. J. Biomol. Eng. 14, 143-149 (1999); Morris T. et al., J. Clin. Microbiol. 34, 2933 (1996); Lyamichev, V. et al., Science, 260, 778-783 (1993) and the like). In the Invader assay, SNP site can be genotyped by hybridization of an allele-specific oligonucleotide and DNA containing SNP to be genotyped. The Invader assay uses two types of unlabeled oligo and one type of fluorescence-labeled oligo. One of the two types of unlabeled oligo is called an allele probe.

An allele probe comprises a 3' hybridizing region which hybridizes to a genomic DNA (template DNA) to form a complementary strand, and a 5' region (called FLAP) which has a sequence unrelated to the sequence of a genomic DNA and does not hybridize to the genomic DNA. A nucleotide located at the 5' end of the hybridizing region corresponds to an SNP (Fig. 4a). That is, an allele probe is provided with the hybridizing region which can form a complementary strand with a region on the 5' side from the SNP site of genomic DNA ("A" in Fig. 4a), and Flap region which has been added to the 5' side of a nucleotide ("T" in Fig. 4a) corresponding to the SNP. Here, the term "Flap" is an oligonucleotide having a sequence complementary to a given region of a FRET probe which is described later.

Another unlabeled oligo is called an Invader probe. The Invader probe is designed so as to complementarily hybridize in a direction from the SNP site ("A" in Fig. 4b) to the 3' end of a genomic DNA (Fig. 4b). Here, a sequence ("N" in Fig. 4b) corresponding to an SNP site may be an arbitrary nucleotide. Thus, hybridization of a genomic DNA as a template and the above two probes causes one nucleotide (N) of the invader probe to invade into the SNP site (Fig. 4c).

A fluorescence-labeled oligo is a sequence totally independent from genomic

DNA, and the sequence is a common sequence regardless of the type of SNP. This fluorescence-labeled oligo is called a FRET probe (fluorescence resonance energy transfer probe) (Fig. 5). A nucleotide (reporter, located at the 5' terminus of FRET probe) is labeled with fluorescent dye (R), and quencher (Q) is bound upstream of the nucleotide. In this condition, no fluorescence can be detected with a fluorescent detector since the quencher absorbs fluorescence.

A certain region (region 1) from the 5' end (reporter nucleotide) of FRET probe is designed to face a region (region 2) on the 3' side of the region 1 so as to form a complementary sequence. Hence, in FRET probe, region 1 forms a complementary strand with region 2 (Fig. 5). Moreover, a region located further on the 3' side of the region forming a complementary strand, that is, the 3' terminal side of region 2 is designed so as to be able to form a complementary strand by hybridizing to Flap of an allele probe (Fig. 5).

The Invader assay uses Cleavase which is an enzyme (5' nucleotidase) having special endonuclease activity to recognize and cleave a specific structure of DNA. Cleavase can cleave the 3' side of an SNP position of an allele probe when a genomic DNA, allele probe and Invader probe overlap three fold at the SNP position. That is when three nucleotides overlap as shown in Fig. 4c, cleavase recognizes a portion at which the 5' end is Flap-shaped, and cleaves the Flap portion. Therefore, cleavase recognizes this structure of an SNP site (Fig. 6a), the allele probe is cleaved at a nucleotide corresponding to the SNP site, and then the Flap portion is released (Fig. 6b).

Next, the Flap portion released from the allele probe binds complementarily to the FRET probe since it has a sequence complementary to that of the FRET probe (Fig. 6c). At this time, the SNP site of Flap invades into the complementary binding site of FRET itself. Again, cleavase recognizes the structure and cleaves a reporter nucleotide having fluorescent dye. The cleaved fluorescent dye becomes unaffected by the

quencher and emits a measurable fluorescence signal (Fig. 6d). In addition, when a nucleotide corresponding to an SNP of allele probe does not match an SNP site, as shown in Fig. 7, the allele probe is not cleaved and Flap is not released since no specific DNA structure, which is specifically recognized by cleavase, is formed. Therefore, in this case almost all the fluorescent dye is still bound to the reporter nucleotide, and can emit any only low fluorescence signal.

Specifically, when an SNP site can be T or C, an Invader probe, an allele probe for T, and a FRET probe in which FAM has been bound to a reporter nucleotide corresponding to SNP is prepared. Separately, an Invader probe, an allele probe for C, and a FRET probe in which VIC has been bound to a reporter corresponding to SNP is prepared. All of them are mixed and the Invader assay is performed. An SNP site is then genotyped by detecting fluorescent intensity of FAM and that of VIC with a fluorescence detector. That is when strong fluorescence of FAM is detected but very low fluorescence of VIC is detected, the SNP can be genotyped as a homozygote (T/T). When fluorescence of both FAM and VIC can be detected, the SNP can be genotyped as a heterozygote (T/C). Further, when strong fluorescence of VIC is detected but very low fluorescence of FAM is detected, the SNP can be genotyped as a homozygote (T/T).

Now, a so-called SniPer method may be used in the typing step. The SniPer method is based on a technique called RCA (rolling circle amplification) method. In the SniPer method, DNA polymerase sequentially synthesizes a complementary strand DNA while migrating on a circular single stranded DNA as a template. According to the method, SNP can be genotyped by measuring the presence or absence of coloring reaction due to DNA amplification (Lizardi, P. M. et al., Nature Genet., 19, 225-232 (1998); Piated, A. S. et al., Nature Biotech., 16, 359-363 (1998)).

Particularly in the typing step, card 1, as shown in Fig. 8a, is preferably used when any of the methods described above is employed. Card 1 comprises numerous

wells 2, arranged in a matrix on a primary surface thereof, wall surfaces 3 which are so formed as to project around each well 2, and a plurality of grooves 4 which are formed between adjacent wells 2. An example of a card 1 that can be used is, but is not limited to, a card 1 comprising a total of 384 wells 2 (24 columns x 16 lines) formed thereon.

When the typing step is performed using card 1, first the DNA fragment obtained in the above-mentioned amplification step is dispensed into wells 2, in the form of PCR reaction solution. Then, the dispensed PCR reaction solution is dried up, so that the dried DNA fragments remain on the bottom surfaces of wells 2.

Subsequently, as shown in Fig. 8b, a reagent necessary for the typing step, for example, a reagent necessary for the Invader assay is dispensed into wells 2. At this time, the reagent necessary for the typing step is dispensed onto the upper portion of a wall surface 3 in a volume exceeding that of well 2 but not such that it overflows, due to surface tension. Further, the DNA fragment present in a dried state in well 2 is dissolved in the reagent dispensed into wells 2, necessary for the typing step. When a reagent necessary for the typing step is dispensed into wells 2, a pipette preferably used herein is a non-contact dispensing equipment which is capable of simultaneously dispensing a solution into a plurality of wells 2 arranged on card 1. Since a non-contact dispensing equipment does not contact with the inside of wells 2, such a pipette can prevent contamination from occurring between a plurality of cards 1.

Next as shown in Fig. 8c, plastic plate 5 which is large enough to cover the primary surface of card 1 is overlayed on the primary surface of card 1. Accordingly, the reagent dispensed into wells 2 flows out toward the outside of wells 2. Most of the reagent that has flowed out remains within grooves 4.

Then, card 1 with plastic plate 5 overlayed on the primary surface thereof is ultrasonically treated with a ultrasonic welding equipment, so that plastic plate 5 and card 1 are welded. As specifically shown in Fig. 8d, the upper surfaces of the wall surfaces 3 and the plastic plate 5 are in contact with each other, and welding can be

performed at the contacting area.

As described above, the use of card 1 in the typing step enables prevention of foaming within wells 2 and prevents the reagent dispensed within wells 2 from flowing out even when card 1 is subjected to heat treatment. Therefore, card 1 employed in the typing step can improve detection sensitivity for a fluorescent dye or the like.

Further, use of card 1 provided with well 2 whose volume is 0.6 ml can largely decrease the volume of reagent necessary for the typing step, so as to largely reduce the cost required for SNP typing.

Furthermore, when the typing step is performed using card 1, heat treatment or the like included in the typing step is preferably performed using a thermostat water bath. By using a thermostat water bath, the typing step can be performed using numerous cards 1 simultaneously. For example, since performing the typing step simultaneously using numerous cards 1 is difficult when a thermal cycler is used, a thermostat water bath is preferably used.

3. Effect of the method of the present invention

According to the method of the present invention, a target SNP can be genotyped by amplifying in the amplification step genomic DNA collected or extracted, and using the amplified DNA fragments. Therefore, the method enables typing of several hundreds of thousands of SNP sites even with a small amount of genomic DNA. For example, when 100,000 SNP sites are genotyped, approximately 10 μg of genomic DNA would be required according to an estimation that approximately 0.1 ng of genomic DNA is required per SNP site. Approximately 10 μg of genomic DNA can be extracted from 1.25 ml of peripheral blood collected from a human.

On the other hand, since SNP typing using genomic DNA it self by the Invader assay or the like requires several tens ng of genomic DNA per SNP site, several mg of

genomic DNA must be prepared to genotype 100,000 SNP sites. To obtain several mg of genomic DNA, 500 ml or more peripheral blood must be collected. However, it is actually impossible to prepare genomic DNA in such a volume.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically shows TaqMan probes.

Figure 2 shows the outline of each steps composing the TaqMan PCR method.

Figure 3 schematically shows fluorescence-labeled TaqMan probes.

Figure 4 schematically shows the Invader assay.

Figure 5 schematically shows a FRET probe.

Figure 6 schematically shows the Invader assay.

Figure 7 schematically shows a probe which does not match an allele.

Figure 8 shows a partial cross-sectional view of a card used in the typing step.

Figure 9 is a characteristic figure showing the result of typing SNP No. 1.

Figure 10 is a characteristic figure showing the result of typing by the Invader assay directly using genomic DNA.

Figure 11 is a flowchart of the method for typing described in example 4.

Figure 12 is a characteristic figure showing the result of detecting a signal intensity of VIC/ROX and FAM/ROX in example 4.

EXAMPLE

Now the present invention will be described more specifically, but the technical scope of the invention is not limited by the following examples.

Example 1: Preparation of genomic DNA

Leukocytes were isolated from the peripheral blood collected from a subject who had given informed consent, and genomic DNA was extracted therefrom. Genomic DNA was extracted according to Laboratory Manual for Genomic Analysis (Yusuke Nakamura ed., Springer-Verlag Tokyo) as described below. 10 ml of the blood was transferred to a 50 ml Falcon tube, and then centrifuged at 3,000 rpm for 5 min at room temperature. The supernatant (serum) was discarded with a pipette, 30 ml of RBC lysis buffer (10 mM NH_4HCO_3, 144 mM NH_4Cl) was added thereto. After mixing to disperse the precipitate well, the mixture was allowed to stand at room temperature for 20 min. Subsequently, centrifugation was performed at 3,000 rpm for 5 min at room temperature, the supernatant (serum) was discarded with a pipette, thereby obtaining a leukocyte pellet. 30 ml of RBC lysis buffer was added to the pellet, and then a similar procedure was performed twice. 4 ml of Proteinase K buffer (50 mM Tris-HCl(pH 7.4), 100 mM NaCl, 1 mM EDTA(pH 8.0)), 200 μl of 10%SDS, 200 μl of 10 mg/ml Proteinase K were added to the leukocyte pellet. The mixture was mixed by inverting, and allowed to stand at 37℃ overnight. 4 ml of phenol was added to the mixture, and then mixed by slowly inverting for 4 hours with a rotator (Rotator T-50, Taitec). Centrifugation was performed at 3,000rpm for 10min at room temperature, and the upper layer was collected in a new tube. 4 ml of phenol-chloroform-isoamyl alcohol (volume ratio 25:24:1) was added to the product, followed by two hours of similar mixing by inverting and centrifugation. The upper layer was collected in a new tube, and 4 ml of chloroform-isoamyl alcohol (volume ratio 24:1) was added thereto. Then the product was mixed by inverting similarly for 30 min, followed by centrifugation.

The upper layer was collected in a new tube, and 400 μl of 8 M ammonium acetate and 4 ml of isopropanol were added thereto, followed by mixing by inverting. White, filamentous precipitate (DNA) was collected in a 2 ml tube, 1 ml of 70% ethanol was added thereto, followed by mixing by inverting. DNA was collected in a new 2 ml tube and then air-dried. DNA was dissolved in 500 μl of TE solution (10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH7.4)), thereby preparing a genomic DNA sample.

Example 2: Amplification of genomic DNA

PCR was performed with a 50 μl system using 40 ng of the genomic DNA obtained in Example 1. A reaction solution contains 200 types of primer (50 pmol each, 100 pairs, SEQ ID NOS: 1 to 200), 10 units of EX-TaqDNA polymerase (Takara Shuzo), and 0.55 μg of TaqStart (CLONTECH Laboratories). TaqStart is an antibody for EX-TaqDNA polymerase. The hot start method can be performed by adding TaqStart to the reaction solution.

PCR was performed with GeneAmp PCR system 9700 (Applied Biosystems). After DNA was denatured at 94℃ for 2 min, a cycle consisting of a denaturation process at 94℃ for 15 sec, an annealing process at 60℃ at 45 sec, and then an extension process at 72℃ for 3min was repeated 35 times, followed by extension at 72℃ for 3min.

As shown in Table 1, a plurality of DNA fragments containing SNP Identification Nos. ("SNP ID" in Table 1) 1 to 100 can be amplified simultaneously.

Table 1

SNP ID	Forward primer	Reverse primer	SNP name
1	SEQ ID NO1	SEQ ID NO2	AC000353.27_20000214_5_24737
2	SEQ ID NO3	SEQ ID NO4	AC000388.1_19970529_9_37703
3	SEQ ID NO5	SEQ ID NO6	AC001643.1_19970529_3_6293
4	SEQ ID NO7	SEQ ID NO8	AC002237.1_19970606_1_1204
5	SEQ ID NO9	SEQ ID NO10	AC002319.1_19980203_3_29222
6	SEQ ID NO11	SEQ ID NO12	AC002364.1_19981204_2_117944
7	SEQ ID NO13	SEQ ID NO14	AC003005.1_19971022_1_2731

8	SEQ ID NO15	SEQ ID NO16	AC003005.1_19971022_3_5667
9	SEQ ID NO17	SEQ ID NO18	AC003689.1_19981121_2_45471
10	SEQ ID NO19	SEQ ID NO20	AF066064.1_19980603_1_563
11	SEQ ID NO21	SEQ ID NO22	AF077374.1_19990202_1_1708
12	SEQ ID NO23	SEQ ID NO24	AF157101.1_19990624_1_618
13	SEQ ID NO25	SEQ ID NO26	AF196968.1_19991109_1_6368
14	SEQ ID NO27	SEQ ID NO28	AJ009610.1_19990104_4_26810
15	SEQ ID NO29	SEQ ID NO30	AJ011772.1_19981005_2_903
16	SEQ ID NO31	SEQ ID NO32	AJ011931.1_19981110_5_23638
17	SEQ ID NO33	SEQ ID NO34	AJ229043.1_19990122_1_3475
18	SEQ ID NO35	SEQ ID NO36	AL008633.1_19971029_1_33923
19	SEQ ID NO37	SEQ ID NO38	AL008634.1_19981109_13_92880
20	SEQ ID NO39	SEQ ID NO40	AL008634.1_19981109_13_93343
21	SEQ ID NO41	SEQ ID NO42	AL008634.1_19981109_14_95554
22	SEQ ID NO43	SEQ ID NO44	AL008638.1_19981123_4_52385
23	SEQ ID NO45	SEQ ID NO46	AL008730.1_19980204_2_66080
24	SEQ ID NO47	SEQ ID NO48	AL008733.10_19991225_1_5608
25	SEQ ID NO49	SEQ ID NO50	AL008734.10_19990610_1_7867
26	SEQ ID NO51	SEQ ID NO52	AL021917.1_19980721_19_77217
27	SEQ ID NO53	SEQ ID NO54	AL021937.1_19990303_93_124364
28	SEQ ID NO55	SEQ ID NO56	AL022721.1_19990324_13_73842
29	SEQ ID NO57	SEQ ID NO58	AL023279.1_19990305_3_69539
30	SEQ ID NO59	SEQ ID NO60	AL049557.19_73359
31	SEQ ID NO61	SEQ ID NO62	AL049569.13_164971
32	SEQ ID NO63	SEQ ID NO64	AL049569.13_61322
33	SEQ ID NO65	SEQ ID NO66	AL049569.13_61680
34	SEQ ID NO67	SEQ ID NO68	AL049569.13_61971
35	SEQ ID NO69	SEQ ID NO70	AL049569.13_62026
36	SEQ ID NO71	SEQ ID NO72	AL049569.13_87106
37	SEQ ID NO73	SEQ ID NO74	AL049569.13_87279
38	SEQ ID NO75	SEQ ID NO76	AL049569.13_88461
39	SEQ ID NO77	SEQ ID NO78	AL049569.13_88502
40	SEQ ID NO79	SEQ ID NO80	AL049575.7_10509
41	SEQ ID NO81	SEQ ID NO82	AL049611.24_75054
42	SEQ ID NO83	SEQ ID NO84	AL049611.24_75895
43	SEQ ID NO85	SEQ ID NO86	AL049612.11_45784
44	SEQ ID NO87	SEQ ID NO88	AL049649.4_93434
45	SEQ ID NO89	SEQ ID NO90	AL049649.4_93918
46	SEQ ID NO91	SEQ ID NO92	AL049650.8_62150
47	SEQ ID NO93	SEQ ID NO94	AL049691.17_64637
48	SEQ ID NO95	SEQ ID NO96	AL049694.9_4336
49	SEQ ID NO97	SEQ ID NO98	AL049698.3_3216
50	SEQ ID NO99	SEQ ID NO100	AL049698.3_3822
51	SEQ ID NO101	SEQ ID NO102	AL049758.11_67143

52	SEQ ID NO103	SEQ ID NO104	AL049758.11_79044
53	SEQ ID NO105	SEQ ID NO106	AL049759.10_111608
54	SEQ ID NO107	SEQ ID NO108	AL049795.20_113584
55	SEQ ID NO109	SEQ ID NO110	AL049829.2_138544
56	SEQ ID NO111	SEQ ID NO112	AL049829.2_161140
57	SEQ ID NO113	SEQ ID NO114	AL049843.18_49141
58	SEQ ID NO115	SEQ ID NO116	AL096766.12_13162
59	SEQ ID NO117	SEQ ID NO118	AP000065.1_58129
60	SEQ ID NO119	SEQ ID NO120	AP000168.1_56285
61	SEQ ID NO121	SEQ ID NO122	AP000171.1_87106
62	SEQ ID NO123	SEQ ID NO124	AP000347.1_81990
63	SEQ ID NO125	SEQ ID NO126	AP000349.1_19017
64	SEQ ID NO127	SEQ ID NO128	AP000350.1_10554
65	SEQ ID NO129	SEQ ID NO130	AP000350.1_10756
66	SEQ ID NO131	SEQ ID NO132	AP000350.1_11294
67	SEQ ID NO133	SEQ ID NO134	AP000350.1_31581
68	SEQ ID NO135	SEQ ID NO136	AP000352.1_63635
69	SEQ ID NO137	SEQ ID NO138	AP000353.1_86203
70	SEQ ID NO139	SEQ ID NO140	AP000355.1_132012
71	SEQ ID NO141	SEQ ID NO142	AP000493.1_129114
72	SEQ ID NO143	SEQ ID NO144	AP000495.1_60416
73	SEQ ID NO145	SEQ ID NO146	AP000500.1_113211
74	SEQ ID NO147	SEQ ID NO148	AP000500.1_113401
75	SEQ ID NO149	SEQ ID NO150	AP000500.1_194483
76	SEQ ID NO151	SEQ ID NO152	AP000500.1_25277
77	SEQ ID NO153	SEQ ID NO154	AP000501.1_137357
78	SEQ ID NO155	SEQ ID NO156	AP000501.1_99530
79	SEQ ID NO157	SEQ ID NO158	AP001041.1_6501
80	SEQ ID NO159	SEQ ID NO160	AP001041.1_6582
81	SEQ ID NO161	SEQ ID NO162	AP001054.1_35804
82	SEQ ID NO163	SEQ ID NO164	AP001054.1_36083
83	SEQ ID NO165	SEQ ID NO166	AP001054.1_36142
84	SEQ ID NO167	SEQ ID NO168	AP001101.1_12400
85	SEQ ID NO169	SEQ ID NO170	D42052.1_7718
86	SEQ ID NO171	SEQ ID NO172	D50561.1_1218
87	SEQ ID NO173	SEQ ID NO174	D50561.1_564
88	SEQ ID NO175	SEQ ID NO176	NT_002717.1_29435
89	SEQ ID NO177	SEQ ID NO178	U07563.1_68521
90	SEQ ID NO179	SEQ ID NO180	X56832.1_2826
91	SEQ ID NO181	SEQ ID NO182	X69299.1_1633
92	SEQ ID NO183	SEQ ID NO184	X74107.1_29168
93	SEQ ID NO185	SEQ ID NO186	X74107.1_29545
94	SEQ ID NO187	SEQ ID NO188	X78901.1_1934
95	SEQ ID NO189	SEQ ID NO190	X87344.1_115764

96	SEQ ID NO191	SEQ ID NO192	X91863.1_2477
97	SEQ ID NO193	SEQ ID NO194	Y08378.1_1560
98	SEQ ID NO195	SEQ ID NO196	Y12852.1_4439
99	SEQ ID NO197	SEQ ID NO198	Y16792.1_4230
100	SEQ ID NO199	SEQ ID NO200	Z54246.1_8005

Example 3: Typing by Invader assay

The sample obtained by PCR as described in Example 2 was apportioned, 0.2 μl each, to 100 tubes and then typing was performed for 100 types of SNPs using an Invader assay kit (Third Wave Technology). That is, 0.5 μl of the sample was added to the kit containing 0.5 μl of signal buffer, 0.5 μl of FRET probe, 0.5 μl of structure-specific deoxyribonuclease, and 1 μl of allele-specific probe. The reaction volume was prepared to be 10 μl. FRET probes were labeled with different fluorescent dyes (FAM and VIC). Two types of FRET probes differing in their Flap complementary sequences were used. A pair of probes has Flap portions corresponding to two types of FRET probes. Next, the reaction solution was incubated at 95℃ for 5 min, and then 63℃ for 15 min using ABI7700 (Applied Biosystems). Fluorescence emitted during incubation was detected using the device.

Figure 9 a, b and c show respectively the results of typing three different samples using SNP ID NO: 1 probe. In Fig. 9, a continuous line denotes fluorescence of FAM, and a broken line denotes that of VIC. As shown in Fig. 9a, only the fluorescence of VIC was elevated for this sample. This result suggested that both alleles of nucleotides (SNP ID NO: 1) in this sample corresponded to a specific probe having a Flap complementary to FRET probe labeled with VIC, that is, the sample was homozygous. The result for the sample in Fig. 9b suggested that one of the alleles corresponded to a specific probe having a Flap complementary to FAM-labeled FRET probe, and the other allele corresponded to a specific probe having a Flap complementary to VIC-labeled FRET probe, that is, this sample was heterozygous. Further, the sample in Fig. 9c was shown to be homozygous corresponding to a specific

probe having a Flap complementary to FAM-labeled FRET probe.

Moreover, fluorescence was detected for 98% of SNPs (SNP ID NOS: 1 to 100). The result suggested that with a very small amount of genomic DNA, 0.4 ng per SNP, typing was possible by the method of the present invention.

When the Invader assay was directly performed using 0.4 ng of genomic DNA, no fluorescence was detected and typing was impossible as shown in Fig. 10. Probably, this was due to the amount of DNA (0.4 ng) used for assay being insufficient to obtain fluorescence required for detection.

Example 4 Improved method

A typing system using a smaller quantity of genomic DNA was studied by improving the typing system described in Example 3. In this example, a 96-well PCR plate was used and 96 DNA fragments were amplified in each well with a single amplification reaction. In addition, the flow chart of the typing system performed in this Example 4 is shown in Fig. 11.

The PCR product obtained in Example 2 was diluted and then transferred into a 384 deep well. Then 0.8 μl of the PCR product was dispensed into each well (volume: 0.6 μl) of a card shown in Fig. 8a. An automatic liquid handling system, Tango (Robbins), was used for dispensing. The PCR product was dispensed using an automatic liquid handling system Tango from a single plate having 384 deep wells to 96 cards. The automatic liquid handling system Tango is capable of simultaneously dispensing 384 samples, that is, capable of dispensing into 8 cards in a single operation. Thereafter, the dispensed PCR product was naturally dried at room temperature.

Next, 0.03 μl of signal buffer, 0.03 μl of FRET probe, 0.03 μl of structure specific deoxyribonuclease and 0.06 μl of allele specific probe contained in an Invader

assay kit (Third Wave Technology) were dispensed into wells. These solutions were dispensed using a non-contact dispensing workstation PixSys4200 (Cartesian Technologies). The non-contact dispensing workstation PixSys4200 is capable of simultaneously dispensing the above solutions into 8 cards, each having 384 wells.

Subsequently, a plastic plate was overlayed on the card, and then ultrasonic welding using ultrasonic welding equipment (Branson) was performed. Thus, 96 cards, each having 384 wells, could be prepared. Then, incubation was performed using a thermobath (TAITEC) at 95℃ for 5 min, followed by 63℃ for 60 min. After incubation, fluorescence emitted from each well of the cards was detected using a fluorescence detector ABI 7900(Applied Biosystems), and typing was performed. Fig. 12 shows the result detecting a signal intensity of VIC/ROX and FAM/ROX. In Fig. 12, the horizontal axis shows the signal intensity of VIC/ROX; the vertical axis shows the signal intensity of FAM/ROX. The bottom right cluster of spots indicates a strong VIC/ROX signal and a weak FAM/ROX signal; the samples in this cluster are judged to be homozygous for allele 1. Similarly, samples indicated by the top right cluster of spots are heterozygous, and samples indicated by the top left cluster of spots are homozygous for allele 2.

Although only 0.1ng genomic DNA was used as template, alleles could be discriminated clearly.

According to Example 4, as shown in Fig.12, alleles could be discriminated clearly and fluorescence could be detected with high sensitivity even when 0.1 ng of the genomic DNA was subjected to a single typing, thereby allowing accurate typing. Thus, genomic DNA to be subjected to a single PCR reaction in an amplification step would be approximately 10 ng. Therefore according to Example 4, with genomic DNA in a volume approximately 1/4 of that used in the method of Example 3, typing can be performed.

Effect of the invention

As described above in detail, the method for SNP typing according to the present invention can type several hundreds of thousands of SNP sites using a very small amount of genomic DNA. Hence, the method for SNP typing according to the present invention enables typing of several hundreds of thousands of SNP sites using very small amount of genomic DNA at low cost and in a short period of time.

SEQUENCE LISTING FREE TEXT

SEQ ID NOS: 1 to 200 are synthetic primers.